Date: 01/16/02 Express Mail Label No. EL930598879 US

Inventors: David M. Knipe, Travis J Taylor and Elizabeth Emily McNamee

Attorney's Docket No.: 0725.1059-001

REPLICATION-COMPETENT VIRUS EXPRESSING A DETECTABLE FUSION PROTEIN

RELATED APPLICATION

This application is a continuation of U.S. Application No. 09/127,227, filed July 31, 1998. The entire teachings of the above application are incorporated herein by reference.

GOVERNMENT SUPPORT

The invention was supported, in whole or in part, by a grant CA26345 from Genetics of Herpesvirus Transformation. The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Viruses play an important role in a person's health. Several viruses infect people for which no effective treatment plan exists. Examples of such viruses include herpesvirus, Human Immunodeficiency Virus (HIV) and the common cold. Finding effective anti-viral drugs depends on the ability to track the virus in a host or cell after the drug has been administered. Therefore, a need exists to be able to efficiently detect the presence or absence of a virus. A further need exists to develop screening methods for determining the efficacy of anti-viral agents or compounds. Another need exists to determine whether a cell is resistant to viral infection.

SUMMARY OF THE INVENTION

The invention embodies a fusion protein comprising a viral protein and a detectable protein. The viral protein and the detectable protein can be linked so that viral protein's native function is maintained. The detectable fusion protein can efficiently detect the presence, absence, or amount of viral DNA replication and, therefore, infection. The protein is thus a marker selected from a variety of viruses. Examples of such viruses are: the retrovirus (Human Immunodeficiency Virus (HIV)), the influenzavirus, the papillomavirus, the rhinovirus, and the herpesvirus (e.g., Herpes Simplex Virus-1 (HSV-1), Herpes Simplex Virus-2 (HSV-2), a Varicella-Zoster Virus, Epstein-Barr Virus, Cytomegalovirus, Human Herpesvirus-6, and Human Herpesvirus-7). In particular, a claimed invention embodies a viral protein from a Herpesvirus fused with a detectable protein (e.g., a fluorescent protein, or a protein that emits fluorescence upon excitation). An example of a fluorescent protein which can be used in the invention is a green fluorescent protein (GFP). In particular, a preferred embodiment utilizes the HSV ICP8 viral protein fused with a fluorescent protein, such as ICP8-GFP. In another embodiment, the invention pertains to antibodies which selectively bind to such a fusion protein, and plasmids or vectors that encode the fusion proteins, as described herein.

The invention also encompasses a virus that comprises a nucleic acid which expresses the claimed fusion protein. The recombinant virus which expresses the fusion protein is generally able to perform functions similar to a corresponding wild-type virus. The virus is preferably replication competent and in the case of the Herpesvirus able to form replication compartments, a "factory" where DNA synthesis takes place and possibly where virions are assembled. The invention embodies a virus which can express a fusion protein that consists of a viral protein and a fluorescent protein. In particular, the claimed invention relates to a virus that expresses ICP8-GFP.

The invention also includes methods for determining whether a cell is virus resistant. Such a method includes infecting the cell to be tested with a virus that expresses the fusion protein, and then detecting the presence or absence of the fusion

protein. A fusion protein comprising a fluorescent protein can be identified by detecting the amount of fluorescence emitted by the protein. The invention also pertains to a method for identifying an anti-viral agent or an agent that blocks infection or other viral function and, thus, the expression of the fusion protein. Such a method can involve infecting a host cell with a virus as described herein and subjecting a host cell to the agent to be tested, and then detecting the presence of the fusion protein. A decrease in the amount of fusion protein indicates that the agent can be an anti-viral agent or an agent that blocks the expression of the fusion protein. Again, when the fusion protein contains a fluorescent protein, the amount of virus present can be detected by the amount of fluorescence emitted by the fusion protein. Another way of detecting the amount of herpesviral replication present can be by determining the amount of replication compartment formation.

The invention also embodies identifying an agent that reduces infection of a virus *in vivo*. Such a method includes infecting a mammal with a virus that expresses the fusion protein and then subjecting the mammal with the agent to be tested. One then removes a portion of the infected tissue and detects the amount of fusion protein that is produced. A decrease in the amount of fusion protein indicates that the agent is effective in treating the virus. Similarly, the invention can be used to assay for virus resistant cells.

The claimed invention specifically embodies a fusion protein having a herpesviral protein (e.g., ICP8), and the fluorescent protein (e.g., a green fluorescent protein (GFP)). The ICP8-green fluorescent protein is embodied by the claimed invention and its amino acid sequence is referred to herein as SEQ ID NO: 2. The claimed invention also relates to the nucleic acid sequence that encodes the ICP8-GFP fusion protein and is referred to herein as SEQ ID NO: 1.

The invention also relates to a kit that comprises a virus which is capable of expressing the fusion protein, as described herein. The kit can further comprise a complementing cell line or one that expresses the corresponding wild-type viral protein (e.g., S-2) and/or a cell line into which the virus can be transfected (e.g., Vero cell).

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing features and advantages of the invention will be apparent from the following more particular description of the preferred embodiments of the invention, as illustrated in the accompanying figures. Emphasis is placed on illustrating the principles of the invention.

Figure 1 is a diagram illustrating the pICP8-GFP. The figure maps the residues of the ICP8-GFP sequence.

Figure 2 shows the single cycle growth of the 8GFP recombinant virus, as determined by a plaque assay on S2 cells.

Figures 3A, 3B and 3C illustrate the localization of 8GFP in cultured S2 cells. Figure 3A shows mock infected cells. Figure 3B shows S2 cells at 7.5 hours post infection. Figure 3C shows S2 cells with 400 ug/ml PAA at 7.5 hours post-infection.

Figures 4A and 4B demonstrate the expression of 8GFP in murine cornea at two days post-infection. Figure 4A is at 400x magnification of infected cornea and Figure 4B shows the phase at the same magnification.

Figures 5A-5D show the expression of 8GFP in murine trigeminal ganglia neurons at four days post-infection. Figures 5A-5D are panels at 1000x magnification.

Figures 6A-6C show the nucleic acid sequence that encodes for the ICP8-GFP fusion protein (e.g., SEQ ID NO: 1).

Figures 7A-7D show the amino acid sequence of the ICP8-GFP fusion protein (e.g., SEQ ID NO: 2).

DETAILED DESCRIPTION OF THE INVENTION

The invention embodies a fusion protein comprising a viral protein and a detectable protein. The fusion protein generally maintains some function of the native viral protein, however, the function can be maintained to a lesser or greater extent. The specific function of the protein to be selected is not generally critical.

The viral protein can be derived from a variety of viruses. Such viruses include the retrovirus (e.g., Human Immunodeficiency virus (HIV)), influenzavirus, rhinovirus,

papillomavirus and herpesvirus. A preferred embodiment of the invention encompasses a viral protein from a herpesvirus. Examples of herpesviruses are Herpes Simplex Virus-1 (HSV-1), Herpes Simplex Virus-2 (HSV-2), Varicella-Zoster Virus (VZV), Epstein-Bar Virus (EBV), Cytomegalovirus (CMV), Human Herpesvirus-6 (HHV-6), and Human Herpesvirus-7 (HHV-7). In particular, a preferred embodiment of the invention includes a viral particle from the Herpes Simplex Virus (HSV). An embodiment includes the HSV ICP8 viral protein as a fusing partner.

Another portion of the fusion protein, as described above, is the detectable protein. Preferably, the protein can be detected visually or fluorescently. The detectable protein can be detected automatically or may require excitation. A preferred embodiment of the invention employs a fluorescent protein as the detectable protein. The fluorescent protein is defined as a protein that emits fluorescence upon excitation. Therefore, a preferred embodiment of the claimed invention is a Herpesvirus protein fused with a fluorescent protein. An example of a fluorescent protein that is utilized by the claimed invention is the green fluorescent protein (GFP). In particular, an embodiment of the claimed invention is a fusion protein linking ICP8 and GFP, referred to as "ICP8-GFP." ICP8-GFP has an amino acid sequence, SEQ ID NO:2, and is shown in Figures 7A-D. The invention also encompasses the nucleic acid sequence that encodes for ICP8-GFP, designated as SEQ ID NO:1, shown in Figures 6A-C.

The term "protein" is intended to encompass fragments, analogs or derivatives of the native protein. Generally, the fragment, analog or derivative maintains at least one function. The claimed invention is intended to embody the various functional domains of the fusion protein, as described herein. Analogous amino acid sequences generally mean amino acid sequences with sufficient identity to the native protein amino acid sequence so as to possess the biological activity of the fusion protein. For example, an analogous peptide can be produced with "silent" changes in amino acid sequence wherein one, or more, amino acid residues differ from the amino acid residues of the fusion protein, yet still possess substantially the same biological activity of the native. Examples of such differences include additions, deletions or substitutions of residues of

the amino acid sequence of the fusion protein. Also encompassed by the claimed invention are analogous polypeptides that exhibit greater, or lesser, biological activity of the fusion protein.

The claimed invention also encompasses biologically active polypeptide fragments of the fusion protein described herein. Such fragments can include only a part of a full length amino acid sequence of fusion protein and yet possess the same function, possibly to a lesser or greater extent. For example, polypeptide fragments comprising deletion mutants of the fusion protein can be designed and expressed by well known laboratory methods. Such polypeptide fragments can be evaluated for biological activity.

Forms of Nucleic Acid that Encodes the Fusion Protein

The claimed invention encompasses isolated nucleic acid sequences encoding the fusion protein, and fragments of nucleic acid sequences encoding biologically active portions of the fusion protein.

Fragments of the nucleic acid sequences described herein are useful as probes to detect the presence of the nucleic acid that encodes the fusion protein. Also encompassed by the claimed invention are nucleic acid sequences, DNA or RNA, which are substantially complementary to the DNA sequences encoding the fusion protein, and which specifically hybridize with the DNA sequences under conditions of stringency known to those of skill in the art. Substantially complementary means that the nucleic acid need not reflect the exact sequence of the fusion protein DNA, but are sufficiently similar in sequence to permit hybridization with the fusion protein DNA under stringent conditions. Conditions for stringency are described in e.g., Ausubel, F.M., *et al.*, Current Protocols in Molecular Biology, (Current Protocol, 1994).

The invention also provides vectors or plasmids containing the nucleic acid that encodes for the fusion protein. Suitable vectors for use in eukaryotic and prokaryotic cells are known in the art and are commercially available or readily prepared by a skilled

artisan. Additional vectors can also be found, for example, in Ausubel, F.M., *et al.*, Current Protocols in Molecular Biology, (Current Protocol, 1994) and Sambrook *et al.*, "Molecular Cloning: A Laboratory Manual," 2nd ED. (1989), the teachings of which are incorporated herein by reference.

The nucleic acid molecule can be incorporated or inserted into the host cell by known methods. Methods for preparing such recombinant host cells and incorporating nucleic acids are described in more detail in Sambrook *et al.*, "Molecular Cloning: A Laboratory Manual," Second Edition (1989) and Ausubel, *et al.* "Current Protocols in Molecular Biology," (1992), for example. Once the nucleic acid is incorporated into the host cell, the cell can be maintained under suitable conditions for expression and recovery of fusion protein. Generally, the cells are maintained in a suitable buffer and/or growth medium or nutrient source for growth of the cells and expression of the gene product(s). The growth media are not critical to the invention, are generally known in the art and can include sources of carbon, nitrogen and sulfur. Examples include Luria broth, Superbroth, Dulbecco's Modified Eagles Media (DMEM), RPMI-1640, M199 and Grace's insect media. The growth Media may contain a buffer, the selection of which is not critical to the invention. The pH of the buffered media can be selected and is generally one tolerated by or optimal for growth for the host cell.

A Virus that Expresses the Fusion Protein

The invention relates to a virus comprising a nucleic acid which expresses the fusion protein, as described herein. The virus generally corresponds to the viral protein used in the fusion protein. For example, if a viral protein from HSV-1 is fused with a fluorescent protein, then the HSV-1 virus can be used to express this fusion protein. A virus that expresses the fusion protein is generally replication competent. The term, "replication competent," is defined as the ability for the virus to grow and replicate viral DNA and, optionally, in normal cultured cells (e.g., a Herpesvirus that grows in a Vero cell) infect or spread to new cells. This can include the ability to make infectious viral particles and infect new cells. Specifically, for herpesviruses, the virus can include an

ability to form replication compartments. The formation of replication compartments refers to a factory where DNA synthesis takes place, and possibly the area where virions are assembled. A virus that expresses the fusion protein and maintains functions similar to the corresponding wild type virus or maintains one or more "viral functions" also generally includes the ability for the virus to undergo attachment, transcription, DNA replication, and/or assembly including the ability to infect a host cell. The claimed virus that expresses the fusion protein should exhibit at least one of these functional characteristics, although these functional characteristics may independently occur to a lesser or greater extent. In particular, a preferred embodiment of the invention is a Herpesvirus that expresses the fusion protein (e.g., a fusion protein containing a Herpesvirus protein fused with a detectable protein). Specifically, the claimed invention embodies a virus that expresses the ICP8-GFP fusion protein. This virus is referred to as the "8GFP virus."

The invention also pertains to a kit comprising the virus which is described herein. The kit may also include a cell line that is capable of complementing one or more viral proteins. For example, the 8GFP virus may be accompanied by S-2 cells, a complementing cell line which expresses HSV ICP8. A kit may further comprise a cell line into which the virus may be transfected, e.g., a vero cell.

Construction Of The Fusion Gene And A Virus That Can Express The Fusion Protein

Construction of a gene that encodes a fusion protein requires routine methods and techniques. Ausubel, F. M., *et al.*, "Current Protocols in Molecular Biology," John Wiley & Sons (1998); Sambrook, *et al.*, "Molecular Cloning: A Laboratory Manual" 2nd Edition (1989). A skilled artisan must link two nucleotide sequences so that the open reading frame is maintained. Using the appropriate restriction sites, one can clone the nucleic acid sequence of one of the proteins to be fused (e.g., the detectable protein) into a vector. Subsequently, the nucleic acid sequence of the second protein (e.g., the viral protein) may also be cloned into the same vector in a manner to maintain the open

reading frame and link the nucleotide sequences of both proteins. This is done by appropriately utilizing restriction sites and enzymes. Thereafter, PCR is used to amplify the sequence. The reaction is then digested and a sufficient amount of sequence is transfected into an expression system (e.g., *E. coli*).

The detectable protein may be fused or linked to either the amino terminus or the carboxyl terminus of the viral protein. A preferred embodiment of the claimed invention pertains to a Herpesvirus viral protein fused with a fluorescent protein. In particular, the preferred embodiment of the invention relates to the ICP8-GFP fusion protein. The ICP8 viral protein is derived from an a herpesvirus, such as HSV-1 virus. In this case, the fluorescent protein was linked to the carboxyl terminus of the viral protein, which allowed the viral protein (e.g., ICP8) to maintain its function (e.g., its role in viral replication). Other herpesvirus proteins which can be used are described in Field's Virology:(Vol 1 & 2), Fields, Bernard N., et al., editor, Lippincot-Raven Publishers, Philadelphia, PA (1996). the contents of which are incorporated herein by reference in their entirety. A description of how to construct the fusion protein is discussed throughout the specification and in particular in Example 1. Although Example 1 illustrates the specific instructions of how to make the ICP8-GFP fusion protein, these methods and materials can be adapted to make the fusion protein from any viral protein and/or detectable protein.

Construction Of A Virus That Expresses The Fusion Protein

Construction of a virus that expresses a fusion protein as described herein, also requires well known molecular cloning techniques. Ausubel, F. M., *et al.*, "Current Protocols in Molecular Biology," John Wiley & Sons (1998); Sambrook, *et al.*, "Molecular Cloning: A Laboratory Manual" 2nd Edition (1989). A skilled artisan can combine the fusion protein's nucleic acid with a corresponding mutated virus. This virus is mutated so that viral protein that was used for the fusion protein, has been altered or otherwise nonfunctional or ineffective. In the preferred embodiment, the inventors combined the ICP8-GFP fusion protein with a virus that expressed a truncated

form of ICP8. The defective virus and the fusion protein were cotransfected into a complementing cell line, a cell line which expresses the native viral protein (e.g., ICP8). The cells are then grown and cultured. The cells are screened by detecting the detectable protein. The preferred embodiment of the claimed invention includes a Herpesvirus that can express a Herpesvirus protein fused with a fluorescent protein. Accordingly, in constructing such a virus, the presence of the virus was assayed by detecting the emission of fluorescence using an ultraviolet light.

Another preferred embodiment of the invention is the 8GFP virus, which expresses the ICP8-GFP fusion protein. This virus was constructed using a HD-2 replication defective mutant virus. HD-2 makes an altered, nonfunctional form of ICP8, truncated ICP8. This mutant virus was cotransfected with the nucleic acid that encodes the ICP8-GFP fusion protein. One can make a virus in which an altered viral protein is expressed using known methods in the art and methods detailed in the following patent applications: Serial Nos. 09/034,464, 08/903,830, 08/278,601, 08/179,106, and 07/922,912, entitled "Herpesvirus Replication Defective Mutants", the teachings of which are incorporated herein by reference in their entirety. Example 1 describes in more detail the methods and materials necessary to construct the 8GFP virus. The methods described in Example 1 can be modified and adapted by methods known in the art to make other viruses that express a fusion protein, including other Herpesviruses, retroviruses, papillomaviruses, influenzaviruses, and Rhinoviruses.

Applications Of A Replication Competent Virus That Expresses A Fusion Protein

The novelty of the claimed invention lies with the ability to readily and easily detect a virus that can undergo similar functions of the corresponding wild type virus. Such a virus enables a skilled artisan to more easily study the virus and drugs that may impact the virus's ability to infect a host cell or replicate. The claimed invention embodies such a virus. A preferred embodiment is a Herpesvirus that is detectable by virtue of the expression of a detectable fusion protein, as described herein.

This virus allows a skilled artisan to screen for virus resistant cells and identify antiviral drug targets. The replication competent virus also enables one to assay for antiviral agents or compounds that block expression of the detectable protein or its assembly into replication compartment in cultured cells. Furthermore, this virus allows for the assessment of the efficiency of antiviral agents or immunological reagents *in vivo*.

Methods for screening infection in cells involve contacting the virus with the cell under conditions that are sufficient for the virus to infect the cell. Determining such conditions are matters that are routine to a skilled artisan. A skilled artisan can detect the presence or absence of the fusion protein. In the preferred embodiment, the detectable protein is a fluorescent protein. Therefore, detecting the absence or presence of the fusion protein can involve detecting the amount of fluorescence emitted by the fusion protein. A cell that contains the fusion protein will emit fluorescence, whereas a cell that does not contain the fusion protein will not emit fluorescence. A cell which does not emit fluorescence likely does not contain the virus and is, therefore, not infected. Contrarily, a cell that contains the fusion protein also contains the virus, and is, therefore, infected. These cells can be sorted with a fluorescence activated cell sorter. The cells that are resistant can be grown and cultured, a process that can be repeated more than once. Repetition of the process ensures that you have obtained a virus resistant cell and its progeny also provides virus resistant cells. Another approach for detecting virus resistant cells involves selecting for cells which survive. After selecting surviving cells, a skilled artisan can measure the fluorescence emitted from the cells. Cells which survive and do not emit fluorescence are considered to be virus resistant cells. The assay can be used, therefore, to detect virus-resistant and/or virus-susceptible cells.

The methods can be used for the identification of antiviral drug targets, antiviral agents or compounds, and/or agents that block the expression of the fusion protein. These methods are performed in a similar manner described above, adding the agent to be tested. Preferably, the methods involve contacting a virus, as described herein, with

the cell, under conditions that are sufficient to allow the virus to infect the cell. The agent to be tested can be added with, before or after the addition of the virus or cell. The amount of fusion protein or virus present can be detected in a number of ways. In the preferred embodiment, wherein the detectable protein is a fluorescent protein, the fusion protein can be detected by detecting the amount of fluorescence emitted. A decrease in the amount or absence of fluorescence indicates that the agent has antiviral activity. The fluorescence can be measured against a control (e.g., a virus that expresses the fusion protein without being subjected to the agent) or compared to an appropriate standard for the assay. Another way for identifying an antiviral agent or an agent that blocks the expression of the fusion protein is by analyzing the replication compartment formation by the virus. A lack of replication compartment formation indicates interference with the viral cycle, such as occurs in blocking DNA replication or decreasing the assembly of virions.

Another way of identifying an antiviral agent or an agent that blocks expression of the fusion protein is by subjecting the cells which have been infected with the virus and allowing the virus to spread in the presence of the agent. A skilled artisan can then detect the spread of fluorescence emitted by the cells. Again, a decrease in fluorescence indicates that the agent is an antiviral agent or one that blocks expression of the fusion protein.

A replication competent virus that expresses a detectable fusion protein can also be used in the methods for identify an agent that reduces the infection of a virus *in vivo*. The method involves infecting a mammal with the virus that expresses the fusion protein and then subjecting the mammal with the agent to be tested. A portion of the infected tissue can then be removed and analyzed. A skilled artisan can detect the amount of the fusion protein that is expressed by the virus as described herein. Again, a decrease in the amount of fluorescence indicates that the agent can reduce infection of the virus *in vivo*.

The claimed invention embodies any antiviral agent, as identified by the methods described herein. An antiviral agent is defined as a compound, drug,

immunological reagent or composition, that can reduce or cease the viral infection, or symptoms that are associated with viral infection.

EXEMPLIFICATION

Example 1. Replication-competent herpesvirus expressing a fluorescent nuclear protein

This invention reports a replication-competent herpes simplex virus that expresses a viral DNA replication protein fused to and tagged with the green fluorescent protein (GFP). The fluorescent fusion protein enters the nucleus and assembles into replication compartments. Among other applications, this virus can be used as: 1) a means to screen for virus-resistant cells and to identify new antiviral drug targets, 2) an assay for antiviral compounds that block expression of the fluorescent fusion protein or its assembly into replication compartments in cultured cells, or 3) an assay for antiviral compounds or immunological reagents that reduce viral infection and spread in experimental animal systems such as the mouse cornea.

Construction of an ICP8-GFP fusion gene. A 732 base pair NotI fragment containing the green fluorescent protein (GFP) open reading frame from pGreenLantern (Gibco-BRL) was cloned into the NotI site of pCIΔAflII creating pCIΔA-GFP. A 3.8-kb EcoRI/AvrII fragment containing the entire ICP8 ORF from pSV8.2 was cloned into the EcoRI and XbaI sites of pCIΔA-GFP. The fusion protein ORF was generated by mutating the ICP8 stop codon via PCR mediated site-directed mutagenesis with *Pfu* DNA polymerase (Stratagene) (one cycle 95° C 2 min; twelve cycles 95° C 30 s, 55° C 1 min, 68° C 18 min). The two primers used for changing the stop codon to an arginine residue were 5'CAACCCCTCTCAGCATATCCAACG-3' (SEQ ID NO:3) (sense) and 5'CGTTGGATATGCTGAGAGGGGTTG-3' (SEQ ID NO:4) (antisense) (Gibco-BRL). The nucleotide that was altered is underlined. After PCR amplification, the reaction was digested with DpnI, and 1 μl of the reaction was used to transform *E. coli*. The fusion protein consists of 1453 amino acid residues: 1196 residues from ICP8, 18

residues in the linker region between ICP8 and GFP, and 239 residues from GFP (Figure 1). The predicted size of the ICP8-GFP fusion protein is approximately 160 kiloDaltons (kDa).

The EcoRI site 172 nucleotides upstream from the ICP8 gene ATG was filled in and converted to a BglII restriction site by the insertion of a 12 base pair oligonucleotide linker (5' -GGAAGATCTTCC-3') (SEQ ID NO:5) (NEB). The HpaI site 147 nucleotides downstream of the GFP coding sequence was also converted to a BglII restriction site with the same oligonucleotide linker. The resulting 4.7-kb BglII fragment was cloned into the BglII site of pICP8PA, which contains the ICP8 promoter and poly A sequences, creating pICP8-GFP (see Figure 1). The ICP8 promoter requires other viral proteins for expression so, upon transfection of Vero cells with pICP8-GFP, the cells with not efficiently express the ICP8-GFP fusion protein unless subsequently superinfected with HSV.

Isolation of a recombinant HSV-1 expressing the ICP8-GFP fusion protein. To create an ICP8-GFP expressing virus via homologous recombinants (Fig. 1), linearized pICP8-GFP and HD-2 viral DNA were co-transfected via the calcium phosphate method into the ICP8 complementing cell line V827. HD-2 is a replication defective virus that expresses a truncated ICP8 fused to the lacZ ORF (ICP8-lacZ). The DNA was transfected with various molar ratios of HD-2 viral DNA to plasmid DNA (1:10, 1:15, 1:20) and brought to a total of 16 µg DNA with salmon sperm DNA. An equal volume of 2X HBS pH 7.05 (270 mM NaCl, 10mM KCl, 1.4 mM Na2HPO4, 2% dextrose, 42 mM HEPES) was added to the DNA. The volume was brought up to 600 µl with lX HBS, and then 40 µl of 2.0 M CaC12 was added dropwise. The tubes were mixed gently and incubated at room temperature for 15 min. The precipitate was then added to T25 flasks of subconfluent V827 cells and incubated at room temperature for 30 min, after which 5 ml of DMEM+ 10% FBS was added and the flasks were placed at 37° C. On the following day, the cells were washed twice and overlaid with new media. Four days later, the cells were harvested by adding 2.5-ml sterile milk and freeze-thawing twice. The lysate was sonicated on ice for 30 seconds on/30 seconds off three

times. The lysate was then serially diluted and plated on V827 cells. An inverted, fluorescent Nikon microscope equipped with a FITC filter set was used to screen for fluorescent plaques under UV light. A glowing plaque was chosen and plaque purified twice more on V827 cells for the preparation of a stock of the ICP8-GFP virus.

Characterization of the ICP8-GFP recombinant virus. To confirm that the recombinant ICP8-GFP virus expressed the correct fusion protein, labeled cell extracts were analyzed by SDS-PAGE. Briefly, Vero cell monolayers were infected at a multiplicity of infection (moi) of 10 with the recombinant ICP8-GFP virus, wildtype KOS 1.1 virus, or the parental HD-2 virus. The cells were labeled for 30 minutes at 4, 6, 8, 10, or 12 hours post-infection and subsequently harvested in lysis buffer (60mM Tris-Cl, pH 7.5, 2% SDS, 20% glycerol, 0.5% BME, 2 μg/ml aprotinin, 5 μg/ml leupeptin). Labeled, infected cell proteins were separated by SDS-PAGE. The gel was fixed, dried, and exposed to Kodak Bio-Max MR film. The wildtype ICP8 (128kDa) and the HD-2 ICP8-lacZ (145kDa) proteins were readily detected in the lanes corresponding to KOS 1.1 and HD-2 infections, respectively. Neither wildtype ICP8 nor ICP8-lacZ proteins could be detected in the lanes corresponding with ICP8-GFP virus infection, rather, a new band at the predicted size (160 kDa) of the ICP8-GFP fusion protein was observed. The kinetics of ICP8-GFP expression was similar to that of the wildtype ICP8, but the amount of labeled ICP8-GFP protein was partially decreased. Thus, the recombinant ICP8-GFP virus expresses only the fusion protein and neither the wildtype ICP8 nor the parental ICP8-lacZ proteins.

To determine if the virus could grow in normal cells, single-cycle growth experiments were performed in both an ICP8 complementing cell line (S2) and a noncomplementing cell line (Vero). Monolayers of cells were infected at an moi of 3 or 20 and virus was harvested 24 hours post-infection as described above. The resulting virus was titered on S2 cells. The 8GFP viral yields at an moi 20 were 6.6×10^7 pfu/ml and 4.6×10^5 pfu/ml on the S2 and Vero cells, respectively (see Figure 2). Therefore, the virus can grow on Vero cells, but the yield is increased almost 150 fold by growing the virus on an ICP8-GFP complementing cell line. Thus, the virus appears to be

compromised in Vero cells at some point in the replication cycle. The ICP8-GFP virus can grow to wildtype titers on an ICP8 complementing cell line.

To analyze ICP8-GFP viral DNA synthesis in Vero cells, viral DNA replication assays were performed. Vero cell monolayers in T25 flasks were infected with either KOS 1.1 or the ICP8-GFP virus at an moi of 3 or 10 in the presence or absence of the specific viral DNA inhibitor phosphonoacetic acid (PAA) (400μg/ml). At 16 hours post-infection, total DNA was obtained by lysing the cells in 3 ml lysis buffer (10mM Tris-Cl, pH 8.0, 10mM EDTA, pH 8.0, 2% SDS, 100μg/ml proteinase K) and incubated overnight at 37° C. After the addition of 0.3 ml 3M sodium acetate (pH5.2), lysates were extracted once with phenol:chloroform and then once with chloroform. After precipitation with 2 volumes 70% ethanol and resuspension in 700 μl TE, the lysate was digested RNase A (25 μg/ml) for 30 minutes at 37° C. 70 μl of 3M sodium acetate (pH5.2) was added and then the samples were once again extracted with phenol:chloroform and then once with chloroform. The DNA was precipitated on ice for one hour by the addition of an equal volume of 1.6 M NaCl-13% PEG. The DNA was then resuspended in TE quantitated. Equivalent amounts of DNA were applied to a nitrocellulose filter in five fold dilutions (2000ng-16ng in 12X SSC) via a dot-blotter apparatus. The filter was then probed with HSV-1 specific ^{32}P-labeled plasmid pSV8.3. Exposure and quantification of the filter was with the BioRad GS-525 Molecular Imager and Multi-Analyst software. The values were corrected for the amount of input viral DNA, as measured by the signal from lanes containing PAA. At an moi 3, ICP8-GFP viral DNA synthesis was decreased 33-fold compared to KOS 1.1 infection. The defect was slightly diminished at an moi of 10 with a decrease in viral DNA synthesis of 20-fold compared to KOS 1.1. Therefore, the partial block in viral replication in Vero cells may be at the level of viral DNA synthesis.

To analyze replication compartment formation, Vero or S2 cells grown on 22x22 mm coverslips were infected at an moi of 20 with either the ICP8-GFP virus or KOS 1.1 as a control. At various times post-infection, cells were fixed for 3 minutes in 3.7 % formaldehyde in PBS, and then the coverslips were mounted on slides with

glycerol:gelatin. The ICP8-GFP fusion protein was visualized using a filter set designed to detect FITC staining. By 4-4.5 hours post-infection all cells had glowing nuclei with small replication compartments observed in most Vero or S2 cells infected with the ICP8-GFP virus. At no time was glowing observed in either mock or KOS 1.1 infected Vero or S2 cells. At 6 to 9 hours post-infection, the replication compartments increased in size, but in Vero cells the replication compartments did not grow as large as the compartments observed in the infected S2 cells. Thus, the kinetics of replication compartment formation in ICP8-GFP infection was similar to wildtype infection, but the size of the compartments in Vero cells was smaller than what was observed in the infected ICP8 complementing S2 cells. Replication compartments were also observed in cultured Vero or S2 cells with the use of an inverted, fluorescent Nikon Microscope (see Figure 3). It appears as though the ICP8-GFP fusion protein is targeted to the proper intranuclear regions, but it is compromised for a function that is required for the development of large replication compartments. Therefore, the fusion protein appears to be partially deficient in an activity that is required after correct intranuclear localization, but prior to viral DNA synthesis. This deficiency apparently can be complemented by the presence of the wildtype ICP8 protein since the S2 cells, replication compartment formation was near wildtype in both kinetics and size.

To determine if the recombinant 8GFP virus could be used to observe infected cells *in vivo*, mice were co-infected via corneal scarification with 8GFP and KOS 1.1 at 2×10^6 PFU/eye each. At 2 and 4 days post injection, eyes and trigeminal ganglia were removed, respectively, and processed for visualization of 8GFP by cryo-sectioning. In both corneal and ganglion sections, infected cells could be observed (see figures 4 and 5). Thus, the 8GFP virus may be used to study the in vivo localization of ICP8 or simply to identify HSV-1 infected cells in vivo.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.